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C O N T E N T S

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INFLUENCE OF NITROGEN CONCENTRATION ON THE C-PHYCOCYANIN PRODUCTION OF *Spirulina platensis* (GOMONT) GEITLER

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Abstract: *Spirulina platensis* is a filamentous cyanobacterium that is widely used as a functional food due to its high nutritional value. A 24-day cultivation of *S. platensis* was conducted to assess the influence of varying nitrogen concentration in the production of C-phycocyanin (CPC). The CPC concentration, yield and purity were determined. The result showed that the CPC concentration and yield were highest at day 20 with 0.446 ± 0.057 mg/mL and 293.56 ± 78.33 mg/g, respectively. The purity of CPC extracted was highest at day 16 and considered as food grade with purity value from 0.78 to 0.97. This study showed high biomass productivity on nitrogen source.

Keywords: *Spirulina platensis, nitrogen sources, C-phycocyanin*

1. INTRODUCTION

Spirulina platensis is a filamentous cyanobacterium that is widely used as a functional food because of its ability to produce large scale of valuable products. These commercially cultured microalgae have high nutritional value due to its chemical composition that provides good source of proteins, vitamins, essential amino acids, and fatty acids (Ciferri, 1985). It is known to be as "super food" that can be legally marketed as a food supplement (USFDA, 1981).

The cultivation of *S. platensis* can be influenced by various factors such as temperature, pH, light intensity, and nutrients (Colla et al., 2007). A rich nutrient medium can ensure higher growth rate of the microalgae cells (Kaewdam, 2019). Nitrogen is an important nutrient for cell growth since it is the major constituents of all structural and functional proteins in algal cells (Cai et al., 2013; Hu, 2013). Thus, the use of nitrogen is one of the primary requirements for growing any media for cells, like *S. platensis*. This can be supported by several studies that confirmed the starvation of nitrogen causes stress to microorganisms and increasing the levels of nitrogen concentration will result to a significantly higher biomass production (Filali, 1997; Rafiqul, 2003; Sujathakand, 2012). Moreover, nitrogen is involved in the production of pigments because it is the major component of photosynthetic apparatus as well as in processing essential compounds such as amino acids, chlorophyll, and protein (Colla et al., 2007).

Phycobiliproteins are large water-soluble supramolecular protein that is responsible for light capitations during photosynthesis (Róman et al., 2002). It is mainly divided into

three categories based on their spectral properties: C-phycocyanin (CPC), allophycocyanin (APC) and phycoerythrins (Walter et al., 2011; Silva, 2008). The C-phycocyanin (CPC) is a highly dominant pigment protein found in *Spirulina*. This has many biological activities like anti-inflammatory, hepatoprotective, cholesterol-lowering and antioxidant properties (Romay et al., 2003). Because of the high concentration of CPC in *Spirulina*, this has been widely used for commercial application as a natural blue dye colorant for cosmetics (O'hEocha, 1963) and recently for food items. Moreover, many of the functional food item that are based on algae now confirms to be safe for consumption and has a substantial antioxidant potential and immunomodulatory activities (Grover et al., 2021).

The large-scale production of *Spirulina* depends greatly on nutrient availability aside from temperature and light. These factors influence the growth and composition of the microorganism, specifically the accumulation of biomass components like protein and other cellular components such as phycocyanin (Kim & Young, 2007). Thus, the objective of this study is to investigate the influence of different nitrogen concentration present in different culture medium of *S. platensis* to optimize the growth and production of CPC.

2. METHODOLOGY

2.1 Cultivation of Spirulina

Spirulina platensis was cultivated at Phycology Laboratory, Research Institute for Science and Technology (RIST), Polytechnic University of the Philippines. Cultivation was conducted for 20 days using 1 L glass culture bottles using 800 mL modified Zarrouk's medium, which was prepared by mixing 0.09 % (v/v) of SL1 + 0.09% (v/v) SL2 + 99.80% (v/v) SL3 (Table 1). Sodium nitrate ($NaNO_3$) was used as the source of nitrogen for the culture medium. Adjustments were made to come up with varying nitrogen concentrations as presented in Table 2. The cultures were maintained under laboratory condition with 24-hour illumination using white LED fluorescent light and continuous aeration at ambient temperature.

2.2 Harvesting & lyophilization

Spirulina cultures were harvested by gravimetric method. Cultures were filtered using chiffon fabric (60 µm mesh) to eliminate excess media that have solidified and further passed through a nylon filter (45 µm mesh). The harvested fresh biomass was subsequently lyophilized continuously for 12 hours to 14 hours at approximately -50ºC to -60ºC. This includes 4 hours of freezing the fresh biomass and 8 hours to 10 hours of vacuum drying. After lyophilization, the dried biomass was pulverized using the mortar and pestle, then stored in amber glass container prior to use.

2.3 C-phycocyanin (CPC) analysis

The CPC was extracted in dried biomass (100 mg) of *S. platensis* in 10 mL of Phosphate Buffer Saline (pH 7.4) by sonication at 100% frequency. It was further stored inside a refrigerator for 24 hours and centrifuged at 4000 rpm for 15 minutes. The resulting supernatant was used for the analysis of CPC concentration, yield, and purity.

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Table 1. Composition of modified Zarrouk's medium.

Solution	Component	% Composition (w/w)
Solution 1 (SL1)	H_3BO_3	0.2854
	$MnCl_2 \cdot 4H_2O$	0.1806
	$ZnSO_3 \cdot 7H_2O$	0.0220
	$Na_2Mo_7O_{24} \cdot 4H_2O$	0.0020
	$CuSO_4 \cdot 5H_2O$	0.0080
	sterilized H_2O	99.7875
Solution 2 (SL2)	NH_4VO_3	0.0023
	$K2Cr(SO_4)_4 \cdot 24H_2O$	0.0096
	$NiSO_4 \cdot 7H_2O$	0.0048
	$Na_2WO_4 \cdot 2H_2O$	0.0018
	$Co(NO3)2 \cdot 7H2O$	0.0004
	sterilized H_2O	99.9811
Solution	Component	% Composition (w/w)
Solution 3 (SL3)	$NaHCO_3$	1.6475
	KOH	0.1115
	$NaNO_3$	0.0613
	$MgSO_4$	0.0196
	$CaCl_2$	0.0039
	$FeSO_4$	0.0010
	EDTA	0.0078
	H_3PO_4	0.0276
	H_2SO_4	0.0552
	sterilized H_2O	98.0646

Table 2. Summary of the sodium nitrate used in the culture medium with corresponding nitrogen concentration equivalent.

Treatment	Nitrogen Concentration (g/L)	Sodium Nitrate (g/L)
C1	0.103	0.625
C2	0.206	1.250
C3	0.412	2.500
C4	0.824	5.000
C5	1.648	10.000

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The absorbance of the CPC extract was measured at wavelengths 615 nm and 652 nm using UV-VIS spectrophotometer (Bryant et al., 1979). The CPC concentration was calculated adopting the works of Bennett and Bogorad (1973) by using the formula:

$$CPC\ (mg\ ml^{-1}) = \frac{A_{615} - (0.474\ A_{652})}{5.34}$$

where:

CPC = C-phycocyanin concentration
A = absorbance at wavelengths 615 nm and 652 nm

On the other hand, the CPC yield per dried biomass was calculated by the equation used by Silveira et al. (2007):

$$Yield\ (mg\ g^{-1}) = \frac{(CPC\ Concentration\ x\ Solvent\ Volume\ (mL))}{Dried\ Biomass\ (g)}$$

The purity of CPC was determined by measuring the absorbance of the extract at 615 nm and 280 nm wavelengths using UV-VIS spectrophotometer. It was then calculated by determining the ratio of the absorbances (A_{615}:A_{280}). The purity of the extract is considered as food grade if the resulting ratio is greater than 0.7; reactive grade at 3.9; and analytical grade if more than 4.0 (Silveira et al., 2007).

3. RESULTS AND DISCUSSION

Different cultures of *S. platensis* with varying nitrogen concentration (0.103 g/L to 1.648 g/L) in modified Zarrouks medium were experimented to measure the optimal growth on its chemical composition. The result showed increased growth throughout the 20-day cultivation period. Therefore, this supports the idea that sodium nitrate concentration given to the various treatments provides growth of *S. platensis* (Becker, 1994). The pH of the culture during cultivation period ranges from 10.82 to 10.86 and temperature ranges from 28°C to 35°C. These values are considered as optimal conditions during cultivation that leads to increased algal growth, CO_2 uptake and amino acid content (Torzillo & Vonshak, 1994; Chowdhury, 2005). The requirement of high nitrogen content plus other cultivation condition to produce increasing cellular components supports the study with the results obtained.

CPC was successfully extracted as indicated by the blue-colored extract. This, in general, is the key for successful recovery of the phycobiliproteins in the natural state of *S. platensis* as sonication effectively ruptured the cells thus, released the CPC. *S. platensis* has multi-layered resistant cell walls that makes it difficult to extract (Stewart & Farmer, 1984). Freezing and thawing was considered as best extraction method to obtain higher CPC content (Soni et al., 2008; Bermejo et al., 2006; Sarada et al., 1999; Abalde et al., 1998). However, this study proved that sonication and prolonged exposure to the extracting solvent allowed comparable yield and concentration of CPC. A study

conducted by Moraes (2011) also showed a comparable result using sonication for extraction of CPC in *S. platensis* with 57% efficiency than freeze and thawing.

Figure 1 presents the summary of the concentration, yield and purity of the CPC extracted from *S. platensis* grown in various nitrogen concentrations. Nitrogen is important for a higher synthesis rate of amino acids that would make proteins and cellular components like phycocyanin. An increase concentration will lead to direct proportion on the production of soluble proteins and phycocyanin pigment (Hanna & El-Baky, 2003). In the study, the results showed the CPC concentration ranges from 0.009 mg/mL to 0.446 mg/mL (Figure 1A). The highest CPC concentration (0.446 ± 0.057 mg/mL) was observed in cultures with nitrogen concentration of 0.412 g/L. However, this value showed no significant difference ($0.147 > \alpha_{0.05}$) among the other cultures. On the other hand, the CPC yield (mg/g) obtained from the lyophilized biomass among the cultures range from 32.88 mg/g to 293.56 mg/g (Figure 1B). The highest CPC yield (293.56 ± 78.33 mg/g) was obtained from the cultures treated with 0.824 g/L of nitrogen. The CPC yield obtained from among the cultures also showed no significant differences ($0.843 > \alpha_{0.05}$). The growth rate on *S. platensis* showed similar growth curved among different concentrations but each case has different peak biomass values.

The application of CPC purity in different industries requires different requirements. For food industry, a purity of more than 0.7 is acceptable. In the study, the purity of CPC extracted from all the treatments ranges from 0.78 to 0.97 (Figure 1C). The highest CPC purity (0.97 ± 0.04) was obtained on day 16 from cultures with nitrogen concentration of 0.206 g/L. No significant differences ($0.061 > \alpha = 0.05$) was observed among all treatments.

The different cultures of *S. platensis* treated with varying nitrogen concentration increases in biomass as per observation of its optical density (OD) during the culture period. It started to decline on day 21, suggesting that *Spirulina* already consumed the nutrients present on the medium. Nutrient limitation inhibits the physiological processes of microalgae (Wang et al., 2013). Therefore, the quality of the CPC extracted can be affected during the culture period. It was observed the highest CPC concentration and yield was obtained in cultures with 0.412 (g/L) and 0.824 (g/L) nitrogen, respectively during the day 20 cultivation. However, the purity of CPC is highest (0.97 ± 0.04) at day 16 cultivation at culture with 0.206 g/L nitrogen, which implies food grade purities of CPC (Sivansankari & Ravindran, 2011).

The values obtained for the CPC quality parameters are comparable among the nitrogen treatments. Therefore, the Zarrouk's medium with nitrogen concentration of 0.412 g/L is the best culture condition to achieve higher CPC concentration and yield. It is recommended that cultures be harvested on day 20 that still not compensate the purity of the CPC extracted.

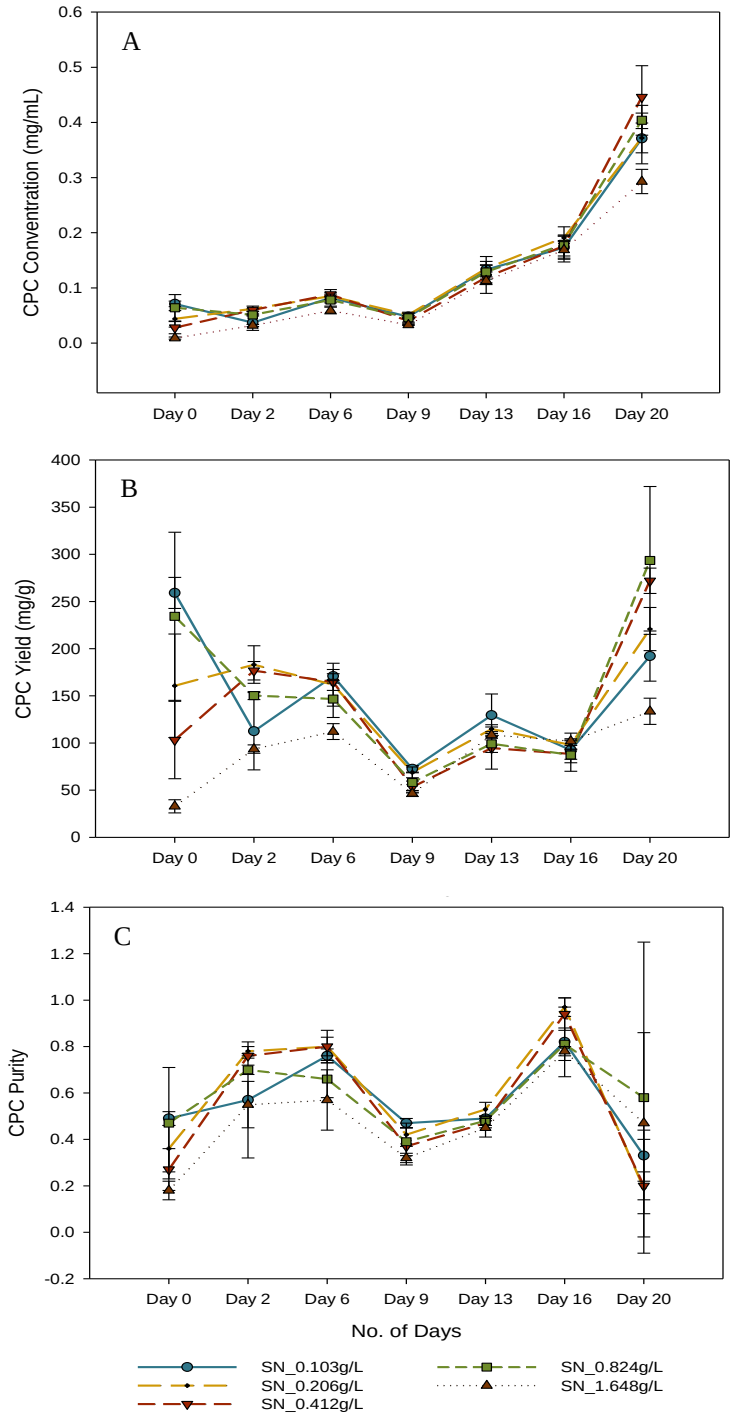

Figure 1. Summary of the CPC concentration, yield and purity produced from *S. platensis* grown in different nitrogen concentrations during the 20-day culture period.

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4. CONCLUSIONS

S. platensis showed efficient and enhanced productivity by using sodium nitrate as its nitrogen source. Although a higher concentration of nitrogen source is the best culture condition for *S. platensis*, a lower nitrogen concentration of 0.412 g/L showed utilization by providing comparable results. The quality of CPC using this culture condition is best harvested at day 20.

5. ACKNOWLEDGMENT

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